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Regulation of Secondary Metabolite Biosynthesis: Catabolite Repression of Phenoxazinone Synthase and Actinomycin Formation by Glucose

MARIA GALLO[1] AND EDWARD KATZ

Department of Microbiology, Georgetown University Schools of Medicine and Dentistry, Washington, D.C. 20007

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Synthesis of the secondary metabolite, actinomycin, and the enzyme, phenoxazinone synthase, involved in the biosynthesis of the antibiotic, were shown to be under severe catabolite repression by glucose. Of a variety of hexoses and carbon compounds examined, glucose, and to a lesser extent, mannose, proved to be the most repressive for enzyme synthesis. The repression by glucose was most evident before production of the antibiotic. In a chemically defined medium suitable for actinomycin production, synthesis of phenoxazinone synthase began at the time the glucose (0.1%) supply was depleted. Soon after, antibiotic synthesis was initiated. Galactose, the major carbon source for growth and antibiotic synthesis, was not utilized until the glucose was consumed. Generally, carbon compounds which supported a rapid rate of growth were most effective in producing catabolite repression.

Actinomycin is a chromopeptide antibiotic synthesized by several species of the genus *Streptomyces*. The molecule consists of a chromophore, actinocin, linked to two cyclic pentapeptide chains (8). Although the peptides may be identical or different in amino acid composition, actinocin is common to all actinomycins examined. The mixture of actinomycins synthesized by *Streptomyces antibioticus* vary only in the imino acid site of the molecule.

Since the initial observations by Epps and Gale (5), the inhibitory effects of glucose and related compounds upon the synthesis of a variety of enzymes by microorganisms have been reported by numerous investigators (17, 24, 25). The repression by glucose of the formation of enzyme systems specifically involved in the biosynthesis of secondary metabolites has been described only recently, however (4, 6, 19).

Marshall et al. (19) observed that the enzyme, phenoxazinone synthase, which catalyzes the synthesis of the actinomycin chromophore, is under catabolite repression during the first 9 hr of growth. Synthesis of the enzyme was shown to begin at 9 hr and to increase markedly (up to 20-fold) by 24 hr. Galactose, glucose, and mannose repressed the increase in enzyme activity. We have examined further the effects of glucose upon synthesis of phenoxazinone synthase and actinomycin by *S. antibioticus* and wish to describe our results in the present communication.

MATERIALS AND METHODS

Chemicals and radioisotopes. L-Valine-*1*-^{14}C (33 mCi/mmole) was purchased from New England Nuclear Corp. 3-Hydroxyanthranilic acid was obtained from Mann Research Laboratories. D-Galactose and D-glucose were purchased from Difco Laboratories and Fisher Scientific Laboratories, Inc., respectively. Chloramphenicol was obtained from Parke, Davis & Co. All other chemicals employed were purchased from commercial sources.

Cultivation of the organism. *S. antibioticus*, strain 3720, was grown at 30 C in 250-ml Erlenmeyer flasks containing 100 ml of a glutamic acid-galactose-mineral salts medium after a 48-hr period of incubation in NZ-amine medium as described previously (9, 12).

Short-term experiments with S. antibioticus. Cultures were taken after 9 hr of incubation in glu-

[1] Present address: Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014.

tamic acid medium, and the mycelium was harvested by centrifugation at 9,000 rev/min. The mycelium was washed twice with 0.85% NaCl and once in the incubation mixture to be employed (19). The mycelium was then resuspended to the original volume in fresh incubation mixture and reincubated, generally for 6 hr at 30 C. The culture suspended in 0.85% NaCl was employed as a control.

Preparation of cell-free extracts. Samples of the culture were removed, centrifuged, and washed once with 0.85% NaCl. The mycelium was suspended in 3 to 4 volumes of cold deionized water and disrupted in a Branson sonifier (Heat Systems Co.) for 2 min in four 30-sec bursts. The disrupted suspensions were then centrifuged at 12,000 rev/min for 20 min in a refrigerated Sorvall RC-2 centrifuge to remove intact cells and cellular debris. The supernatant fluid was decanted and used as the source of phenoxazinone synthase.

Enzyme assay. Enzyme activity was assayed spectrophotometrically with 3-hydroxyanthranilic acid as substrate (11, 13). One unit of enzyme activity is expressed as an optical density change of 1.0 at 452 nm over a period of 5 min at 37 C (11). Cinnabarinic acid, the phenoxazinone formed from 3-hydroxyanthranilic acid, possesses a molar extinction coefficient of 18.0×10^3 at 452 nm. An optical density reading of 0.018 would be equivalent to 1 nmole of product formed.

Determination of mycelial dry weight. Samples of the culture were filtered by suction on tared Whatman no. 42 filter paper (5.5 cm) discs on a Buchner funnel. After being washed four times with 20 ml of distilled water, the papers were placed in petri dishes and dried overnight at 105 C. Upon cooling in a desiccator, the papers were weighed.

Incorporation of ^{14}C-L-valine into cellular proteins and actinomycin. The procedure for harvesting the mycelium and culture filtrate after incorporation of an amino acid into protein and actinomycin was described elsewhere (12). The cells were treated with 5% trichloroacetic acid, ethanol-ether (1:1), and ether; after drying, the protein residue was solubilized by heating in 0.5 N NaOH for 30 min at 100 C.

Culture filtrates (2.5 ml) were extracted with an equal volume of ethyl acetate (12). After centrifugation, the organic layer containing actinomycin was washed with water (5 ml) and then recentrifuged. A sample of the organic layer was employed for scintillation counting.

Analytical methods. Actinomycin formation was assayed by a spectrophotometric procedure (12). Glucose and galactose concentrations were determined by means of the Glucostat and Galactostat assays (1, 26, 27). Galactose oxidase (EC 1.1.3.9) and glucose oxidase (EC 1.1.3.4) were purchased from Worthington Biochemical Corp. Protein was measured by the method of Lowry et al. (16).

Radioactive measurements. Radioactivity measurements were carried out in a refrigerated Nuclear-Chicago liquid scintillation spectrometer with a naphthalene-dioxane solution as described by Bray (2).

RESULTS

Actinomycin synthesis: influence of glucose as sole carbohydrate source. Previous studies showed that maximal synthesis of actinomycin occurred in a chemically defined medium containing galactose (1%), glutamic acid (0.2%), phosphate, and mineral salts (10). In recent years, however, commercially available galactose has proved to be a variable carbon source for actinomycin production. It was found that the addition of 0.1% glucose to the galactose-glutamic acid medium restored actinomycin production to the levels previously obtained. Concentrations of glucose greater than 0.1%, however, were found to reduce actinomycin formation markedly (Diegelmann, *personal communication*).

The influence of varying concentrations of glucose as sole carbohydrate source on antibiotic synthesis is shown in Fig. 1. Virtually no synthesis occurred in a medium containing low concentrations of the sugar (up to 0.1%). Even at higher levels of the hexose (0.5 to 2%), production was considerably less than that observed in the control (1% galactose plus 0.1% glucose) during the entire incubation. Moreover, initiation of antibiotic synthesis was repressed during cultivation in the glucose media. Preliminary analyses indicated that detectable amounts of actinomycin were not produced until most of the glucose was utilized.

Effect of glucose concentration on the initiation of actinomycin synthesis. The influence of glucose concentration upon antibiotic production in a galactose-containing medium is shown in Fig. 2 and Table 1. The data clearly reveal that the initiation of synthesis of actinomycin does not occur until the glucose is virtually consumed. For example, at an initial concentration of 0.015% glucose, detectable amounts of the antibiotic were found at 10 to 12 hr, whereas, at an initial level of 0.1% glucose, production did not begin until after 24 hr of incubation. The time required for utilization of 50% of the glucose and for the initiation of actinomycin formation is shown in Table 1.

Effect of glucose addition at different stages of actinomycin synthesis. In the preceding experiments, glucose was added to the medium at the time of inoculation of the organism. It was of interest to determine its effect when provided at different intervals during the course of growth and actinomycin formation. Glucose (0.5%, final concentration) was supplied at 12, 18, 24, or 36 hr after inoculation to different flasks of *S. antibioticus*. As shown in

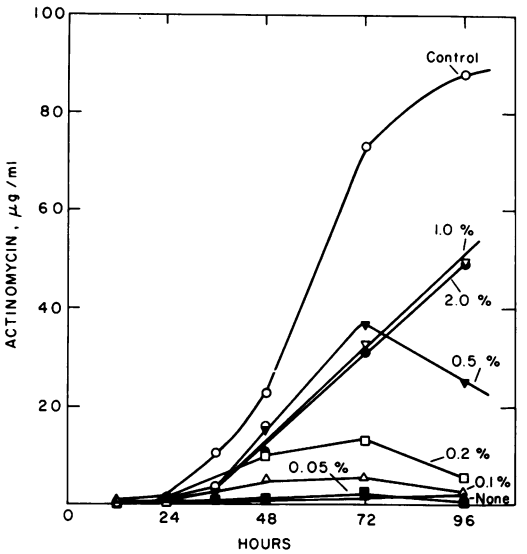

FIG. 1. *Influence of glucose as sole carbohydrate source. Streptomyces antibioticus was grown in glutamic acid-mineral salts medium containing various concentrations of glucose as indicated. Control contained 1% D-galactose and 0.1% glucose.*

Fig. 3, glucose was found to repress actinomycin synthesis when supplied at any of the stated times. Addition of glucose at 12 or 18 hr severely inhibited actinomycin synthesis during the subsequent 6-hr incubation. When it was provided after 24 or 36 hr, however, the repression was of shorter duration, and antibiotic synthesis was resumed.

Effect of multiple additions of glucose upon antibiotic formation. The data in Fig. 1 and 2 indicate that, once glucose is consumed, antibiotic synthesis is initiated. It was reasoned that, if glucose was provided at frequent intervals to a culture to ensure an adequate supply of the hexose, actinomycin synthesis should be repressed throughout a fermentation. The results presented in Table 2 substantiate this view. The data reveal that multiple additions of glucose to the same culture strongly repressed actinomycin synthesis during the 72-hr incubation period. The data are calculated on the basis of actinomycin per milligram of mycelial dry weight rather than per milliliter of medium to compensate for any differences in cell mass and antibiotic formed in the presence of the additional glucose.

Incorporation of L-valine-1-^{14}C into actinomycin and cellular proteins in the presence of glucose. The effect of glucose on actinomycin synthesis was also examined by measuring the incorporation of L-valine-*1*-^{14}*C* into the antibiotic during short-term experiments (12). The incorporation of the labeled amino acid into cellular proteins was also followed. Table 3 reveals that glucose supplied to a 24- or 48-hr-old culture producing actinomycin inhibited antibiotic synthesis. Glucose or a metabolite derived from glucose was effective almost immediately; however, the most pronounced inhibition was observed after 30 min of exposure to the sugar. Incorporation of ^{14}C-labeled valine into cellular proteins (not shown) was not depressed by addition of the hexose; in fact, there was a stimulation (10 to 50%) of incorporation of ^{14}C-isotope into protein. If the inhibition of antibiotic synthesis

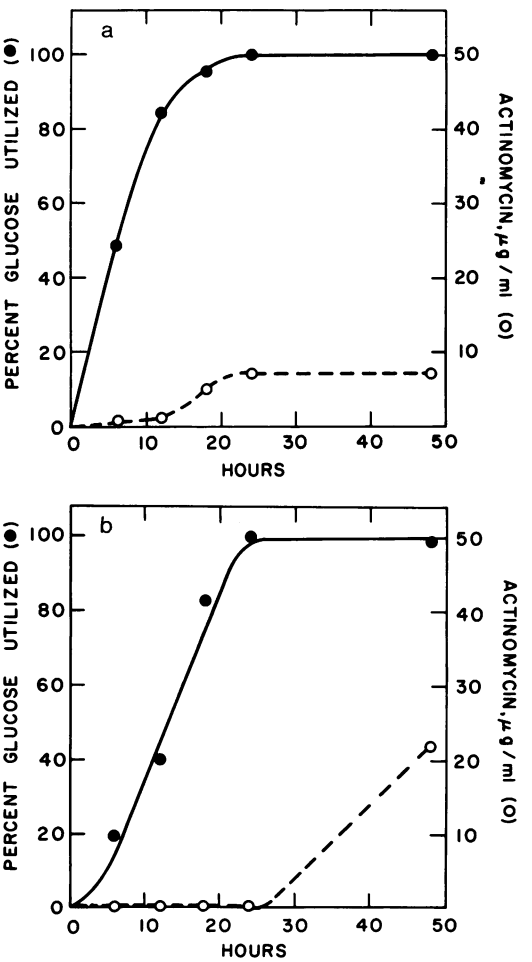

FIG. 2. *Effect of glucose concentration on initiation of actinomycin synthesis. Cultures were grown in glutamic acid-mineral salts medium containing 1.0% galactose and various concentrations of glucose. a, 0.015% glucose; b, 0.1% glucose.*

TABLE 1. *Relationship between utilization of glucose and initiation of actinomycin synthesis*

Initial glucose concn in medium (%)	Time required for 50% consumption of glucose (hr)	Time for initiation of actinomycin synthesis (hr)
0.015	6.0	10–12
0.040	7.2	18
0.070	9.5	18–24
0.100	13.0	24

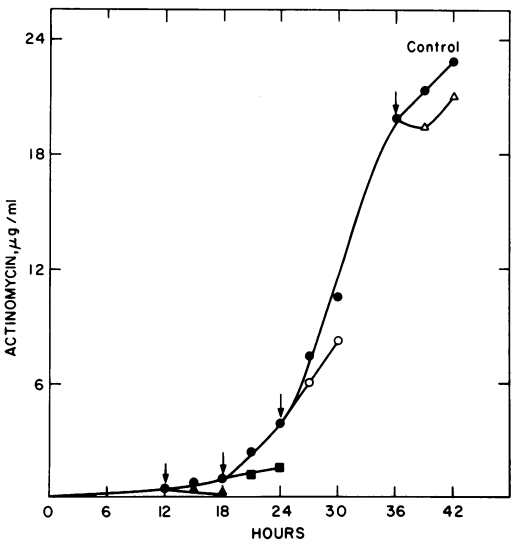

FIG. 3. *Effect of glucose addition at different times during actinomycin synthesis. Cultures were grown in glutamic acid-mineral salts medium containing 1.0% galactose and 0.1% glucose (control, ●). Glucose (0.5%) was added to certain flasks after 12 (▲), 18 (■), 24 (○), or 36 (△) hr of incubation.*

was due to dilution of labeled valine by valine synthesized endogenously from the glucose, one might expect a similar dilution of incorporation of ^{14}C-isotope into cellular proteins.

Comparable results were obtained in an experiment with chloramphenicol present to inhibit protein synthesis. Although incorporation of radiolabel into actinomycin by control cultures was enhanced 1.6- to 1.7-fold by chloramphenicol, as noted previously (12), the addition of glucose was found to decrease antibiotic formation. In this case the most striking effect (35 to 40%) was noted with cultures previously exposed to glucose for 3 hr.

Synthesis of phenoxazinone synthase: effect of glucose concentration. Because glucose inhibited actinomycin synthesis when added before or during antibiotic formation, the effect of the sugar on synthesis of an enzyme which functions in the biosynthesis of antibiotic was also investigated. The inhibition of actinomycin synthesis observed with glucose may be due, in part, to a repression of the synthesis of enzyme(s) that plays a role in antibiotic formation.

The synthesis of phenoxazinone synthase in a galactose-containing medium to which different concentrations of glucose were added is shown in Fig. 4. The lowest glucose concentration employed (0.015%) represents the amount of glucose present as contaminant in the galactose used. It can be seen that the glucose concentration had a marked effect on synthesis of the enzyme during the incubation period. The most severe repression was observed when glucose was employed at levels of 0.1 and 0.5% (not shown).

Glucose addition at intervals during synthesis of phenoxazinone synthase. The data in Fig. 3 demonstrated that glucose, added at 12, 18, 24, or 36 hr, inhibited actinomycin synthesis during the subsequent 6-hr incubation. The effect of glucose on enzyme synthesis was also studied in the same experiment. When glucose was provided after 12, 18, or 24 hr, it was found that enzyme synthesis was almost completely repressed during the subsequent 6-hr incubation period. By contrast, when the sugar was provided at 36 hr, repression of enzyme formation was not observed (Fig. 5).

Relationship of growth, enzyme synthesis, antibiotic formation, and the utilization of glucose and galactose. The early nutritional studies revealed that galactose was an excellent carbon source for actinomycin synthesis, whereas glucose and other hexoses were unsatisfactory for antibiotic production (10). It was shown that glucose was utilized rapidly for

TABLE 2. *Effect of multiple additions of glucose on actinomycin synthesis*[a]

Additions	Actinomycin[b]			
	30 hr	46 hr	60 hr	72 hr
None (control)	2	8	13	25
0.10% glucose	3	1	3	2
0.25% glucose	3	1	3	4
0.50% glucose	2	1	2	3

[a] Culture was grown in glutamic acid-galactose medium containing 0.1% glucose (control). Additional glucose (0.1, 0.25, or 0.5%) was added at 9, 20, and 36 hr to certain flasks. Samples were taken for antibiotic assays at different intervals between 9 and 72 hr.

[b] Antibiotic titer expressed as micrograms of actinomycin per 100 mg of mycelial dry weight.

TABLE 3. *Influence of glucose concentration on the incorporation of* L-*valine-1-*^{14}C *into actinomycin*[a]

Time after exposure to glucose (hr)	Glucose concn (%)	24-hr culture		48-hr culture	
		Actinomycin (dpm/ml)[b]	Inhibition (%)	Actinomycin (dpm/ml)[b]	Inhibition (%)
0	None	0.86		1.73	
	0.1	0.69	21	1.58	8
	0.25	0.65	24	1.35	22
	0.5	0.55	36	1.54	11
	1.0	0.50	42	1.39	20
0.5	None	0.89		1.85	
	0.1	0.59	34	1.24	33
	0.25	0.49	45	1.15	46
	0.5	0.47	48	1.26	41
	1.0	0.40	55	1.34	48
1.0	None	0.87		1.88	
	0.1	0.75	14	1.55	17
	0.25	0.59	33	1.15	39
	0.5	0.66	25	1.26	33
	1.0	0.56	36	1.34	29
3.0	None	1.27		2.10	
	0.1	1.04	18	1.38	10
	0.25	0.97	24	1.32	37
	0.5	0.87	32	1.45	31
	1.0	1.01	20	1.50	28

[a] Cultures were grown for 24 or 48 hr in glutamic acid-galactose (1.0%)-glucose (0.1%)-mineral salts medium. Glucose (0.1, 0.25, 0.5, or 1.0%) was added to flasks, and, after 0, 0.5, 1, and 3 hr of exposure to glucose, 5-ml samples of the culture were removed and incubated with 0.05 ml of L-valine-*1-*^{14}C (1 μCi per 0.5 μmole per ml) for 15 min. Assay procedures are described in the text.

[b] Figures shown to be multiplied $\times 10^3$.

synthesis of mycelium and that there was virtually none left as a carbon and energy source for antibiotic production. In contrast, galactose was consumed slowly; the yield of mycelium was less than that synthesized with glucose, but antibiotic titers were four to six times higher. As described earlier, the variable experience encountered with galactose necessitated a modification of the culture medium, and the addition of glucose (0.1%) to the medium restored antibiotic titers to optimal levels.

It was decided, therefore, to examine in detail the synthesis of phenoxazinone synthase and actinomycin in relation to growth and consumption of the carbon sources by *S. antibioticus*. The data are presented in Fig. 6. Glucose utilization began within 3 to 6 hr of incubation of the culture during the lag phase of growth. Its consumption and the synthesis of mycelium were rapid. By 20 to 24 hr, over 90% of the glucose was metabolized, and 70 to 75% of the mycelium had been synthesized. By 30 hr, the glucose supply was virtually exhausted, and the culture entered the stationary phase. There was very little synthesis of phenoxazinone synthase during the first 20 hr; however, between 20 and 36 hr, the specific activity of the enzyme increased approximately five- to sixfold, and by 48 hr it had increased 12-fold. Detectable amounts of actinomycin were present only after 24 hr. Thus, actinomycin formation lagged somewhat behind the synthesis of the enzyme. Galactose, in contrast to glucose, was consumed very slowly, and it is evident that it was not significantly employed until after the glucose was gone. In fact, there is a lag of several hours before galactose utilization.

Washed-cell experiments. Marshall et al. (19) reported that induction of the enzyme, phenoxazinone synthase, occurred during incubation of mycelium from 9-hr-old cultures of *S. antibioticus* in physiological saline or 0.1 M phosphate buffer, *p*H 7.4. Preliminary studies have confirmed these observations. Marshall et al. (19) noted that galactose, glucose, and mannose repressed synthase formation under these conditions, but their data indicated that glucose and galactose were equally repressive. Our results suggested that glucose was far

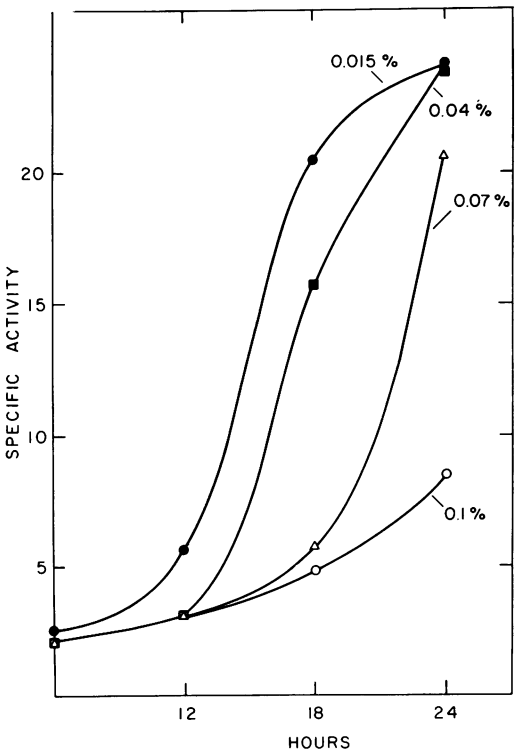

FIG. 4. *Influence of glucose concentration upon phenoxazinone synthase formation. Cultures were grown in glutamic acid-mineral salts medium containing 1% galactose and varying concentrations of glucose. Glucose was added as shown. Cultures were harvested at 6, 12, 18, and 24 hr, and enzyme extracts were prepared as described in text. Specific activity equals optical density units per milligram of protein (11).*

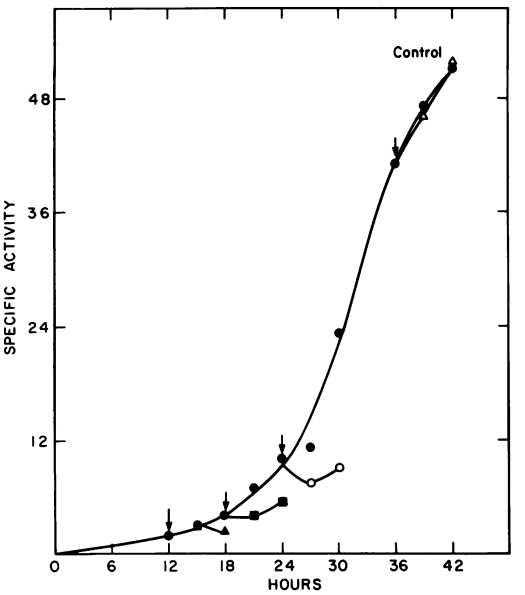

FIG. 5. *Effect of glucose addition at intervals during phenoxazinone synthase formation. Conditions were as described in Fig. 3. Enzyme specific activity equals optical density units per milligram of protein (11).*

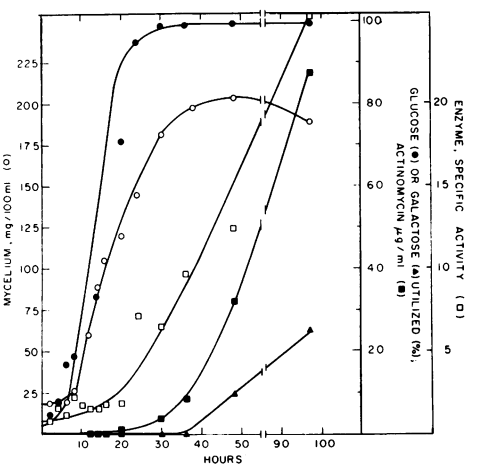

FIG. 6. *Relation of growth, enzyme and antibiotic synthesis, and utilization of sugars by Streptomyces antibioticus. Cultures were grown in glutamic acid-mineral salts medium containing 1.0% galactose and 0.1% glucose. Assays were carried out as described in text.*

more repressive than galactose. In fact, it was observed in different experiments with various levels of galactose that this hexose had only relatively minor effects on enzyme formation.

Effect of various concentrations of glucose and galactose. The influence of varying concentrations of glucose and galactose on enzyme synthesis by washed cells suspended in 0.85% NaCl is shown in Table 4. Glucose, even at 0.01%, markedly repressed enzyme synthesis (70 to 80%). Galactose was also repressive, but the repression of phenoxazinone synthase formation was far less severe than was observed with glucose. As the purified galactose employed contained trace amounts of glucose (0.015%), it is possible that the repressive effect was due to the glucose present as contaminant.

Effect of various carbon sources on enzyme formation by washed cells. Marshall et al. (19) examined the effects of a limited number of carbon compounds on synthesis of phenoxazinone synthase. It was noted that galactose, glucose, and mannose, but not fructose, repressed enzyme formation. A number of carbon compounds were examined in the

TABLE 4. *Influence of glucose and galactose concentration on phenoxazinone synthase formation*[a]

Time (hr)	Addition	Specific activity (units/mg) of protein)	Repression (%)
0	None	2.0	
6	None	11.9	
6	Galactose 0.01%	11.7	2
6	Galactose 0.05%	9.6	23
6	Galactose 0.10%	8.6	33
6	Galactose 0.50%	6.1	58
6	Glucose 0.01%	3.5	85
6	Glucose 0.05%	2.6	94
6	Glucose 0.10%	2.8	92
6	Glucose 0.50%	2.4	96

[a] Mycelia from 9-hr-old cultures were washed with saline as described in the text. Samples of the culture were resuspended in saline with or without glucose or galactose at the concentrations noted. Incubation was for 6 hr. Assays of the galactose used in this experiment revealed a 0.1% contamination by glucose.

present study, and the results (Table 5) showed glucose and mannose to be the most repressive compounds tested. A slight stimulation was observed with sucrose and maltose, whereas a slight repression was found with rhamnose and xylose. The use of galactose, fructose, and lactose, as well as pyruvate, citrate, or acetate, resulted in a somewhat more severe repression of enzyme synthesis. In contrast, succinate and glycerol stimulated enzyme formation under these conditons.

Effect of the carbon sources on growth and enzyme formation. Several of the carbon compounds employed in the previous experiment were examined for their ability to support growth and phenoxazinone synthase formation. The results of this study (Table 6) reveal that an inverse relationship exists between the amount of cellular growth produced and enzyme formation. For example, glycerol yielded the largest increase in mycelial dry weight, but the lowest specific activity. On the other hand, the smallest increase in mycelial dry weight was found with citrate, yet the enzyme exhibited the highest specific activity.

DISCUSSION

Catabolite repression, the inhibition of enzyme synthesis by glucose and related compounds, has been studied extensively. Glucose has been shown to repress the synthesis of both inducible and constitutive enzymes that play a role in the primary metabolism of microorganisms (17, 24, 25). More recently, it has been demonstrated that the hexose inhibits the synthesis of secondary metabolites [e.g., penicillin (3, 7) and siomycin (14, 15)] and certain enzymes [α-D-mannosidase (4, 6) and phenoxazi-

TABLE 5. *Influence of various carbon compounds on phenoxazinone synthase formation*[a]

Time (hr)	Carbon compound	Specific activity (units/mg of protein)	Repression (%)
Expt 1			
0	None	1.8	
6	None	18.3	
6	Glucose	2.8	94
6	Mannose	6.1	74
6	Lactose	11.0	44
6	Fructose	11.4	42
6	Galactose	13.1	31
6	Rhamnose	16.6	10
6	Xylose	16.4	11
6	Maltose	20.3	0
6	Sucrose	21.5	0
Expt 2			
0	None	1.3	
6	None	7.9	
6	Sodium acetate	3.5	69
6	Sodium citrate	4.0	52
6	Sodium pyruvate	4.3	46
6	Glycerol	13.3	0
6	Sodium succinate	18.2	0

[a] Conditions as described under Table 4. Compounds were provided at 0.5% concentration.

TABLE 6. *Effect of carbon sources on growth and phenoxazinone synthase formation*[a]

Time (hr)	Carbon source	Mycelial dry wt (mg/100 ml)	Specific activity (units/mg of protein)
0		8.9	1.8
24	Sodium citrate	22.0	21.3
24	Xylose	96.0	18.6
24	Galactose	122.0	16.2
24	Mannose	138.0	9.9
24	Glycerol	170.0	6.0

[a] Flasks of glutamic acid-mineral salts medium containing the appropriate carbon source (0.5%, final concentration) were inoculated with washed mycelium of *Streptomyces antibioticus* as described in the text. Samples of the culture were taken after 24 hr of incubation for enzyme assay and determination of mycelial dry weight.

none synthase (19)] involved in antibiotic formation.

Marshall et al. (19) noted that the presence or absence of galactose had a profound effect upon the rate of formation of phenoxazinone synthase. A four- to sixfold stimulation of enzyme activity was observed when the cells were incubated in physiological saline or phosphate buffer in the absence of galactose. Repression (38%) of enzyme synthesis was noted with as little as 250 μg of galactose per ml, and the repression increased with an increase in galactose concentration. Glucose and mannose also were found to repress enzyme synthesis. The effect of galactose noted by Marshall et al. (19) may have been due to contamination of the sugar with trace amounts of glucose. Assays carried out during the course of this investigation on a variety of commercial preparations of galactose revealed that glucose is present in amounts ranging from 0.15 to 1.2%.

Of a variety of carbon compounds tested, glucose and mannose produced the most severe repression of phenoxazinone synthase formation. Less severe repression was noted with lactose, galactose, fructose, acetate, citrate, and pyruvate. A comparison of the effect of various concentrations of glucose and galactose revealed that repression of enzyme synthesis was far more severe with glucose (Table 4). The inhibition of actinomycin synthesis by glucose observed in our studies may be related to the inhibition of synthesis of the enzyme(s) that catalyzes the biosynthesis of the antibiotic. The synthesis of phenoxazinone synthase is initiated only when the supply of glucose in the medium has been virtually consumed or under incubation conditions lacking glucose. Actinomycin synthesis begins after enzyme synthesis has started. A direct relationship was shown to exist between the amount of glucose supplied in the medium and the severity of the inhibition of enzyme and antibiotic synthesis. In experiments with growing cells, glucose inhibited both enzyme and actinomycin formation for periods of 6 to 24 hr (Fig. 2a, 2b, and 4). Multiple additions of the hexose almost completely blocked antibiotic formation during a 72-hr incubation period (Table 2). When glucose was added before or during enzyme and antibiotic formation, their syntheses were also repressed. However, the glucose effect was less marked after actinomycin formation had commenced. In fact, no repression of enzyme synthesis was observed after 36 hr of incubation. In short-term experiments, the maximal effect of glucose on antibiotic synthesis (Table 3) was noted after a 30-min incubation with the sugar; the inhibition by glucose was greatly reduced after 3 hr of exposure. The transient inhibition of antibiotic synthesis once actinomycin formation had commenced does not appear to involve enzyme repression to any significant extent but perhaps constitutes inhibition of enzyme activity (catabolite inhibition). Presumably the sugar affects a more sensitive site in actinomycin synthesis. The inhibition may involve a temporary shunt of nutrients and energy supply from secondary to primary metabolism. However, due to the strong competitive requirements of the secondary metabolic systems, a more permanent shift of the cell's activities toward primary metabolism may be precluded. The inability of the cell to shift completely to primary metabolism could be due to the fact that the primary metabolic systems are inactive or functioning at suboptimal rates. Only a carbon compound is being provided when glucose is added; in the absence of an additional nitrogen supply the organism may not be able to reinitiate primary metabolic systems. Some evidence for this view is presented in Tables 5 and 6. With washed cells suspended in saline, glycerol was found to stimulate enzyme synthesis appreciably, whereas citrate was markedly repressive. These, as well as certain other compounds, were tested for their ability to support growth and phenoxazinone synthase formation in a complete medium with glutamate as nitrogen source. An inverse relationship was found to exist between the amount of cellular growth produced on a given carbon compound and enzyme levels present in cell extracts (Table 6). Generally, carbon sources which support a rapid rate of growth were most effective in producing catabolite repression (18, 20–23). The smallest increase in mycelial weight was found with citrate, yet the enzyme exhibited the highest specific activity. On the other hand, the largest increase in mycelial weight and the lowest specific activity were observed with glycerol.

Although the data reveal that glucose can cause a marked repression of phenoxazinone synthase and antibiotic production, the use of a small amount of the hexose (0.1%) in the presence of higher concentrations of galactose is most suitable for antibiotic production under the conditions employed. Presumably, the glucose provides a readily metabolizable compound for the rapid synthesis of cell material. Once the glucose is consumed, the cells begin to form phenoxazinone synthase and presumably the other enzymes required for

actinomycin biosynthesis. Utilization of galactose also appears to be repressed until the glucose is used. Its metabolism provides the carbon and energy for antibiotic production.

It is evident that the enzyme, phenoxazinone synthase, is under catabolite repression by glucose and other carbon compounds. Whether the repression is due to glucose directly or a metabolite derived from glucose is not entirely clear at the present time. The mechanism of the glucose repression of synthesis of phenoxazinone synthase is also not understood. In *Escherichia coli*, the genes involved in the metabolism of lactose have been shown to be under glucose control (25). Repression of β-galactosidase synthesis by glucose can be reversed by cyclic adenosine monophosphate (AMP). The site of action appears to be the promotor region of the *lac* operon. Cyclic AMP has not been tested as yet to establish its effect on the hexose inhibition of synthase formation and antibiotic production. It will be of interest to establish the biochemical factors and genetic determinants which function in actinomycin formation as well as the nature and mechanism of regulation of its biosynthesis.

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